A Role for Glyceraldehyde-3-Phosphate Dehydrogenase in the Development of Thermotolerance in *Xenopus laevis* Embryos

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Abstract. During heat shock, *Xenopus laevis* embryos exhibit an increase in the rate of accumulation of lactate and a loss of ATP relative to non-heat-shocked control embryos. These results suggest that heat shock stimulates a shift in energy metabolism to anaerobic glycolysis while at the same time causing an increase in the demand for ATP. We have evidence indicating that the embryo may meet such demands placed on it by increasing the levels of some glycolytic enzymes. In this report, we show that heat shock stimulates increases in the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase ([EC 1.2.1.12] GAPDH). The specific activity of GAPDH shows a significant increase after heat shock, which correlates with the accumulation of GAPDH in heat-shocked embryos as detected by immunoblotting. Increases in GAPDH-specific activity are variable, however, and are inversely proportional to the levels of specific activity in control embryos; i.e., constitutive enzyme activity. We further analyzed the heat-enhanced accumulation of GAPDH by electrophoretically separating GAPDH isozymes on nondenaturing polyacrylamide gels. Control embryos exhibit a single isozyme of GAPDH, whereas heat-shocked embryos exhibit two isozymes of GAPDH. When these isozymes are labeled with [^{35}S]methionine, separated by nondenaturing gel electrophoresis, and analyzed by fluorography, a heat-shock protein is found to comigrate with the isozyme unique to the heat-shocked sample. Enzyme activity assays at different temperatures suggest that this isozyme has optimum enzymatic activity only at heat-shock temperatures. We have correlated a 35-kD heat-shock protein (hsp35) with GAPDH using the following evidence: this hsp comigrates with GAPDH on one-dimensional SDS polyacrylamide gels; heat-enhanced increases in GAPDH specific activity correlate with hsp35 synthesis; and hsp35 and GAPDH have similar peptide maps. This relationship also provides a compelling explanation for the restriction of hsp35 synthesis to the vegetal hemisphere cells of heat-shocked early gastrulae reported previously (Nickells, R. W., and L. W. Browder. 1985. *Dev. Biol.* 112:391–395).

THERMOTOLERANCE is classically defined as the ability of a cell to withstand a normally lethal heat shock after it has first received a previous treatment at a nonlethal but elevated temperature (28, 32, 40). The term thermotolerance can also be applied to the developmentally acquired ability of an organism to survive heat shock at a temperature that would be lethal to it at early embryonic stages of its development. Thermotolerance, in this respect, is due to normal developmental events that endow the embryo with the capacity to mount a heat-shock response (such as the capacity to transcribe the heat-shock genes), rather than previous exposure to a sublethal heat shock. Examples of organisms that developmentally acquire thermotolerance include *Drosophila* (2, 12, 17), sea urchins (16, 44), and *Xenopus* (20, 21, 39).

The mechanism of thermotolerance may be very complex, because heat shock can cause extensive damage to cells, including disruption of the intermediate filament network (3, 13, 51), changes in membrane fluidity (30; which may be responsible for observed increases in the leakage of small molecules [40] or in the inactivation of intramembranous transport mechanisms [6]), disruption of nucleolar morphology (31, 40, 52), breakage of the DNA (50), potential increases in protein denaturation (reviewed in reference 36), and degradation (42) and disruption of the Golgi apparatus (51) or endoplasmic reticulum (1). In response to heat shock, many cell types synthesize a select group of heat-shock proteins (hsps)[1], some of which may also be constitutively synthesized. Several correlations have been reported between the synthesis of hsps and the development of thermotolerance (see 28, 32 for reviews; also 12, 16, 20, 39). Recently, it has been suggested that the putative role of the 70-kD hsp (hsp70) is to renature denatured proteins (43), such as in the nucleoli of heat-shocked cells (43, 52). These suggestions have been

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1. *Abbreviations used in this paper*: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat-shock protein.

prompted by reports of similar functions for constitutively synthesized proteins related to hsp70 (7, 37, 49).

In addition to structural damage, heat shock also causes changes in cellular energy metabolism. One of the first effects of heat shock on a cell is the rapid loss of ATP (15, 29). Coupled with this is the apparent dysfunction of mitochondria as evidenced by the swelling of these organelles in heat-shocked cells (51), irreversible denaturation of intramembranous mitochondrial proteins (30), and heat-induced inhibition of respiration and oxidative phosphorylation (8, 11, 35). With the inhibition of oxidative phosphorylation and the consequent reduction in ATP production, the cell must shift to alternative sources of energy metabolism, such as anaerobic glycolysis. Such a shift to anaerobic glycolysis is evident in some cells because they are observed to accumulate lactate during heat shock (19, 26). These changes in energy metabolism are also characteristic of heat-shocked *Xenopus* embryos, which, as we demonstrate in this report, rapidly increase the rate of ATP hydrolysis and accumulate lactate during heat shock. The shift to glycolysis may be a vital part of the mechanism of thermotolerance. This possibility is demonstrated by observations of Lindquist et al. (33), who reported that yeast cells growing on dextrose are more thermotolerant than yeast cells growing on acetate. The difference between these two carbon sources is that the metabolism of dextrose occurs via the glycolytic pathway, whereas acetate bypasses these reactions and enters the Kreb's cycle directly. It is conceivable that a dextrose-induced requirement for higher levels of glycolysis makes these cells more suited to withstand heat shock.

The mechanism by which cells can enhance the rate of glycolysis may vary. For example, serum-stimulated quiescent 3T3 cells show increases in glycolytic enzyme activity in the absence of protein synthesis, suggesting allosteric activation of the enzymes (10). Enzyme activity may also be increased by accumulation of enzymes. This is exemplified by heat-shocked yeast cells, which exhibit an increase in the synthesis of the two glycolytic enzymes enolase (23) and glyceraldehyde-3-phosphate dehydrogenase ([EC 1.2.1.12], GAPDH; 32). Because *Xenopus* embryos exhibit heat-induced changes in energy metabolism, we have investigated whether they mount an anaerobic response to heat shock. Here, we report that embryos exhibit increases in the activity and accumulation of a specific isozyme of GAPDH during heat shock. We also present evidence correlating this enzyme with the previously described 35-kD hsp (hsp35) synthesized by heat-shocked *Xenopus* embryos. This hsp is particularly interesting because its synthesis appears to be restricted to the vegetal pole cells of heat-shocked early gastrulae and correlates with the delayed acquisition of thermotolerance of these cells (39).

Materials and Methods

Handling of Animals and Gametes

Sexually mature *Xenopus laevis* frogs were purchased from Nasco Biologicals (Fort Atkinson, WI) and maintained in large holding tanks with continuously flowing dechlorinated water. Gametes were obtained and eggs were artificially fertilized by the methods of Heikkila et al. (20). After fertilization, the eggs were placed in Steinberg's solution (1.4 mM Tris, pH 7.4, 60 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$) and allowed to develop at 22°C. During the incubation of the embryos, the Steinberg's solution was replaced every 10–12 h. At the appropriate developmental stages, the eggs were dejelled as previously described (20).

Embryos were made coenocytic by the method of Newport and Kirschner (38). Eggs were fertilized and then rapidly dejelled. At 40 min postfertilization, the one-cell embryos (first division occurs ~90–100 min postfertilization) were placed on a 50% Ficoll 400 (Pharmacia Fine Chemicals, Dorval, Quebec) cushion and overlayed with a 5% Ficoll solution. In both cases, the Ficoll was dissolved in Steinberg's solution. The embryos were then centrifuged at 22°C at 500 *g* for 10 min. This centrifugation blocks cytokinesis, but not karyokinesis. After centrifugation the coenocytic embryos were washed liberally with Steinberg's solution to remove all traces of Ficoll. Coenocytic embryos were used when control embryos were beginning to gastrulate (~10 h postfertilization). Coenocytic embryos were heat shocked and labeled by incubation in medium containing [^{35}S]methionine.

ATP and Lactate Determinations

The concentrations of ATP and lactate in embryos were determined enzymatically using diagnostic kits available from Sigma Chemical Co. (St. Louis, MO). For sample preparation, groups of thirty embryos were placed in microfuge tubes containing 1 ml of Steinberg's solution. The large volume of Steinberg's solution helped ensure that the samples did not become anaerobic because of the large number on embryos in each tube. These embryos were then heat shocked at 37°C or left at 22°C. At appropriate time intervals, the samples were placed on ice, and the Steinberg's solution was rapidly aspirated and replaced with 300 μl of ice-cold 10 mM K_2HPO_4. The embryos were then homogenized and immediately combined with an equal volume of ice-cold 12% TCA. This mixture was vortexed briefly and incubated on ice 15–30 min. After this incubation, the samples were centrifuged in a microfuge for 10 min at 4°C. The TCA soluble fractions were then assayed immediately as per manufacturer's instructions or were frozen at −70°C until further use.

Heat Shock and Labeling of Embryos

The synthesis of hsp35 is maximal at temperatures above 35°C and during the first 20 min of heat shock (38a). Consequently, for experiments reported here, embryos were heat shocked at 37°C for 20 min unless otherwise stated. For labeling, 10 embryos were placed in microfuge tubes containing [^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Oakville, Ontario) dissolved in Steinberg's solution (45 μCi/100 μl). The embryos were then immediately heat shocked by placing the tubes in a 37°C water bath. After heat shock, the embryos were placed on ice and the labeling medium was aspirated and replaced with ice-cold GAPDH extraction buffer (40 mM Tris, pH 8.0, 111 mM KCl, 0.6 mM EDTA, 6.7 mM Cysteine-HCl; this buffer is designed to stabilize the tetrameric structure of GAPDH [47]) containing heat-denatured pancreatic ribonuclease (to a final concentration of 100 μg/ml). The embryos were homogenized and incubated at 35°C for 5 min. After incubation, the homogenates were centrifuged in a microfuge at 4°C for 10 min. The supernatants were then frozen at −70°C until use. When thawed, the supernatants were cleared by briefly recentrifuging them. No differences were detected in the protein profiles of embryos treated in the same way except with the addition of protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 1 mM Na metabisulphite).

GAPDH Enzyme Assays

Embryos used for enzyme assays were prepared as described above except that they were not labeled with [^{35}S]methionine. The assays were done according to Sullivan et al. (47). Briefly, GAPDH activity was assayed in extraction buffer containing 18.9 mM Na arsenate and 1 mM NAD. The buffer was heated in a 37°C water bath before use. For the assay, 2.5 ml of buffer was added to 10 μl of cleared supernatant in a cuvette and allowed to equilibrate to 37°C for 1 min in a spectrophotometer (model Sp8-150 UV/VIS; Pye-Unicam, Cambridge, England) equipped with a circulating water bath. After equilibration, 0.34 mg of glyceraldehyde-3 phosphate (free acid), available from Sigma Chemical Co. was added to the cuvette, and the linear change in absorbance at 340 mm was measured. The protein concentration of the samples was measured by the Bradford method using commercially prepared stain (Bio-Rad Laboratories, Mississauga, Ontario).

PAGE

For gel elecrophoresis, frozen samples were thawed and cleared. Acid in-

soluble counts for each sample were determined as described by Heikkila et al. (21). For SDS gel electrophoresis, aliquots containing equal acid insoluble counts were removed from each sample and made up to equal volumes with extraction buffer. An equal volume of SDS sample buffer (4% SDS, 20% glycerol, 2% β-mercaptoethanol and bromophenol blue) was added, and the samples were boiled for 3 min. The samples were loaded onto 10% polyacrylamide gels with a 4.5% stacking gel using the buffer system described by Laemmli (27). After electrophoresis, the gels were stained and prepared for fluorography as previously described (20).

Isozymes were separated on 5% polyacrylamide nondenaturing slab gels similar to the method described by Davis (9), using 400 mM Tris, pH 8.9, as the separating gel buffer and 65 mM Tris, pH 6.7, as the stacking gel buffer. The electrode buffer was 5 mM Tris and 38 mM glycine. Gels were run at 20 mA for 3–4 h at 15°C. GAPDH is a basic protein and hence migrates slowly under these gel conditions. Gels were run long enough to adequately separate GAPDH isozymes. As a result, the majority of other proteins (which are less basic) migrate through the gel during an electrophoretic run. To stain for GAPDH activity after electrophoresis, we soaked the gels for 30 min at 37°C in GAPDH extraction buffer (without Cysteine-HCl) containing 18.9 mM Na arsenate and then replaced this buffer with 50 ml fresh buffer containing 6 mg nitro blue tetrazolium, 1 mg phenazine methosulphate, 150 mg NAD, and 3.4 mg of glyceraldehyde-3-phosphate. Staining was allowed to proceed for 1–3 h in the dark at 37°C.

Preparation of Anti-GAPDH Serum and Immunoblotting

Rabbit anti-GAPDH serum was prepared by subcutaneous injection of yeast GAPDH (Sigma Chemical Co.) into New Zealand White Rabbits. For injection, GAPDH was dissolved into PBS (1 mg/ml) and allowed to heat denature for 2 min at 65–70°C. This solution was then mixed with an equal volume of Freund's Complete or Incomplete Adjuvant (Gibco Laboratories, Burlington, Ontario) for the primary or subsequent boosts, respectively. Each rabbit received 0.5 mg of antigen per injection. By boosting the rabbits with antigen, we increased the titer of the antiserum until it primarily recognized *Xenopus* proteins at 35 kD when analyzed by immunoblotting.

For immunoblotting, both SDS gels and nondenaturing gels were run containing equal protein (5–10 μg) loaded for control and heat-shocked samples. After electrophoresis, the polyacrylamide slabs were soaked in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 30 min. The proteins were electrophoretically transferred to prewetted nitrocellulose (0.2 μm pore size; Schleicher & Schuell, Inc., Keene, NH; prewetted in transfer buffer), using a model TE50 apparatus (Hoefer Scientific Instruments, San Francisco, CA). Transfer was complete after 3 h at 300 mA at 4°C. After transfer, the polyacrylamide gels were stained with Coomassie Blue to monitor for complete transfer.

For probing the nitrocellulose with antisera, we first soaked the nitrocellulose overnight at 4°C in PBS containing 5% powdered skim milk. Probing with antiserum was carried out in Sealobags (Philips Electronics, Scarborough, Ontario). The anti-GAPDH serum was diluted in PBS (usually 1:20 or 1:50 dilution) and incubated with the nitrocellulose at 22°C for 2–3 h, after which the filter was washed in PBS (3 × 10 min). Goat anti-rabbit IgG linked to horseradish peroxidase (Amersham Corp.) was used as the secondary antibody. This antibody was used at a dilution of 1:100 in PBS. After incubation with the secondary antibody at 22°C for 1 h and subsequent washing, the filters were stained in PBS containing 0.4 mg/ml diaminobenzidine (Sigma Chemical Co.) and 1 μl/ml 30% hydrogen peroxide.

Peptide Mapping

Partial chemical cleavage of hsp35 and GAPDH was achieved using N'-chlorosuccinimide (Sigma Chemical Co.) according to the method of Lischwe and Ochs (34). Purified rabbit muscle GAPDH (Sigma Chemical Co.) and equal acid insoluble counts of control and heat-shocked samples were subjected to electrophoresis on SDS 10% polyacrylamide gels. The gels were fixed, stained, and destained as described previously. Bands corresponding to hsp35 (from both the control and heat-shock samples) or rabbit muscle GAPDH were cut out of the gel and washed twice in distilled water (10 min each). The slices were then soaked in urea/acetic acid/water (1 g/1 ml/1 ml) for 20 min including one change, followed by incubation in the urea/acetic acid/water mixture containing 15 mM N'-chlorosuccinimide for 30 min at 22°C. The gel slices were then washed twice in distilled water (10 min each) and equilibrated for SDS gel electrophoresis by three incubations in 62.5 mM Tris buffer (pH 6.8) containing 3% SDS, 15% β-mercaptoethanol, and 10% glycerol (20 min each). The slices were loaded into wells of an SDS 4.5% polyacrylamide stacking gel over an SDS 15% polyacrylamide separating gel. In some experiments, a 12% polyacrylamide separating gel was used. After electrophoresis, the gel was stained and prepared for fluorography as described previously (20). Fluorography is necessary to distinguish the fragments of hsp35 from fragments of other proteins that may be present in the same slice of gel. Fragments of hsp35 were considered to be those more intensely labeled in the heat-shock sample relative to the control.

Results

Heat Shock Induces Changes in the Energy Metabolism of Xenopus laevis Embryos

Measurements of the relative levels of ATP and lactate were made on control and heat-shocked neurulae. Fig. 1 illustrates

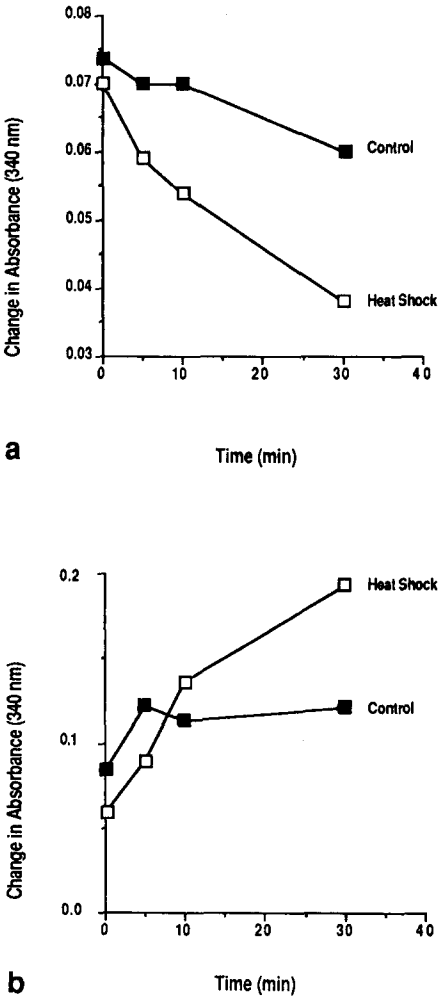

Figure 1. Determination of the relative levels of ATP and lactate in control and heat-shocked neurulae. The neurulae were heat shocked at 37°C for the times indicated. (*a*) ATP concentration was determined enzymatically in a reaction linking phosphoglycerate kinase and GAPDH. In this reaction, 3-phosphoglycerate is converted to glyceraldehyde-3-phosphate by the hydrolysis of ATP to ADP. The concentration of ATP present is directly proportional to the change in NADH to NAD^+, which is monitored by a decrease in absorbance (340 nm). (*b*) Lactate concentration was determined enzymatically using LDH, which converts lactate to pyruvate. The concentration of lactate is directly proportional to the change in NAD^+ to NADH, which is monitored by an increase in absorbance (340 nm).

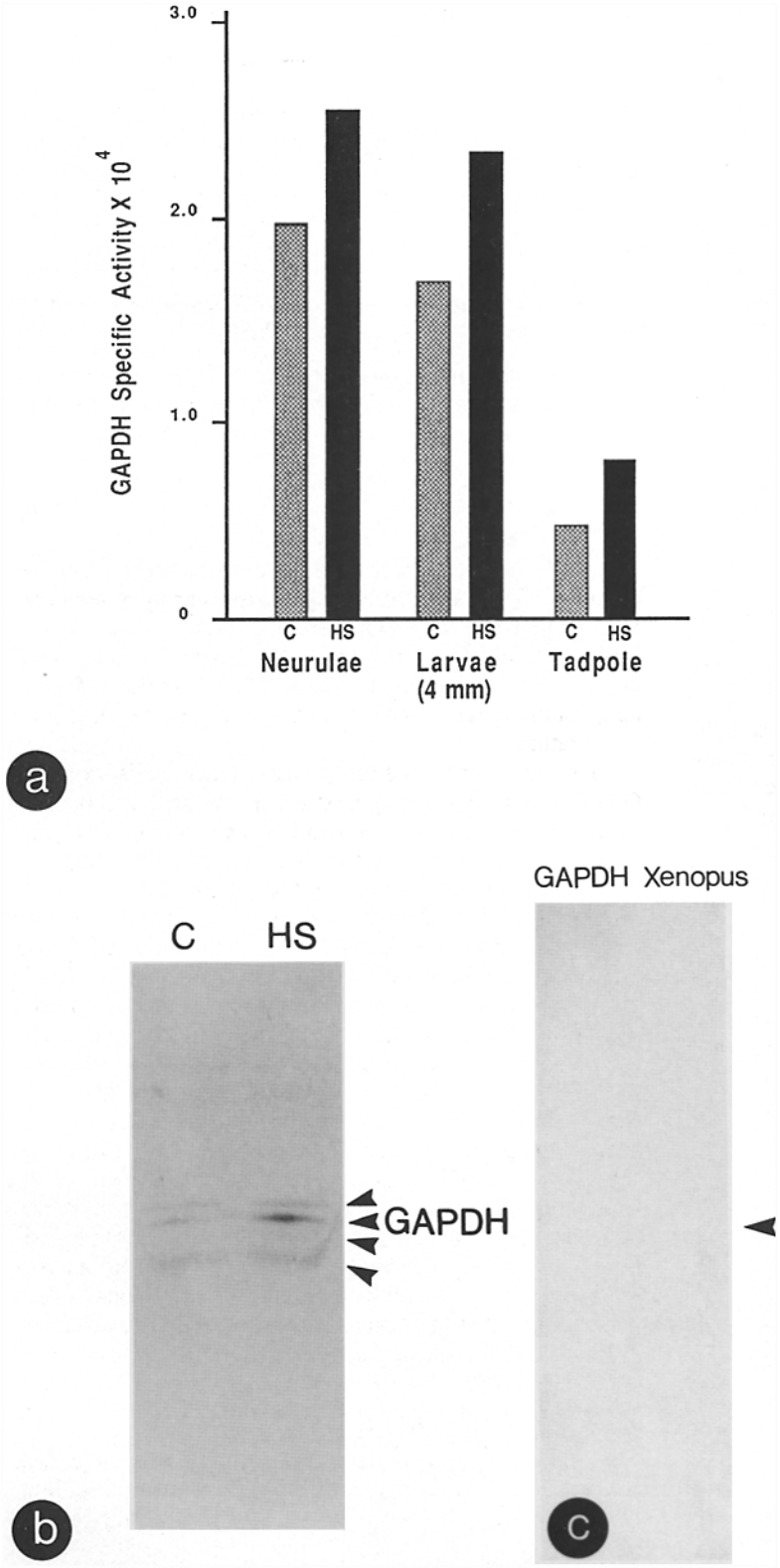

Figure 2. The level of GAPDH increases during heat shock. (*a*) A histogram showing the specific activity of GAPDH in control (*C*) and heat-shocked (*HS*) neurulae and post-neurula stages of development. Specific activity is shown as the mean of four determinations for each sample and is expressed as the linear change in absorbance at 340 nm/s per μg protein. All the heat-shocked samples show a significant increase in specific activity over the respective control samples (Mann Whitney U-test, $p < 0.025$ for each experiment shown). (*b*) Immunoblot of equal amounts of protein from control (*C*) and heat-shocked (*HS*) neurulae probed with GAPDH antiserum. The figure shows a close-up of the immunoblot in the 35-kD region of the gel. The antiserum recognizes four peptides (*small arrowheads*) in each sample. The protein showing the greatest increase during heat shock comigrates with rabbit muscle GAPDH, whose position relative to the *Xenopus* proteins is indicated. (*c*) No cross-reactivity with these *Xenopus* proteins or with pure rabbit muscle GAPDH is detected in a duplicate experiment using preimmune serum.

the relative levels of ATP (*a*) and of lactate (*b*) in neurulae. Both ATP and lactate were determined from the acid soluble lysate of the same sample, with each time point shown on the graphs being the average of duplicate samples. Over a 30-min heat shock, the level of soluble ATP rapidly decreases relative to that of control embryos. It is important to note that after 10 min, control embryos also exhibit a slight decrease in the level of ATP, although the decrease is greater in the heat-shocked embryos. One possible explanation for this result is a loss of viability of the embryos during the course

of the experiment. We have monitored protein-synthetic activity under identical experimental conditions and have found that protein synthesis continues unabated even after 100 min of heat shock at 37°C (Nickells et al., manuscript in preparation), indicating that the effects on viability are negligible. Concomitant with this loss of ATP, the relative level of lactate increases in heat-shocked embryos.

The latter result indicates that heat-shocked *Xenopus* embryos shift into anaerobic glycolysis. It is possible that heat-shocked *Xenopus* embryos synthesize and/or accumulate more of some glycolytic enzymes in an attempt to increase the rate of anaerobic glycolysis to replenish the ATP consumed during heat shock. We have investigated this possibility by examining the effects of heat shock on the levels of GAPDH in *Xenopus* embryos.

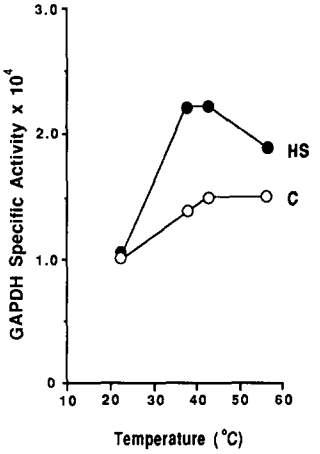

Figure 3. GAPDH in heat-shocked neurulae has a narrow temperature range of activity. Graph showing the temperature curves of GAPDH-specific activity from control and heat-shocked neurulae vs. the temperature used for the assay. The heat-shocked neurulae show a large increase in specific activity only in the temperature range of 37 to 42°C. Enzyme-specific activity is expressed in the same units as shown in Fig 2 *a*.

Increases in GAPDH-specific Activity after Heat Shock Correlate with the Accumulation of GAPDH

We examined the effect of heat shock on the specific activity (i.e., enzyme activity per μg of protein) of GAPDH in a variety of stages of *Xenopus* development. Fig. 2 *a* shows the GAPDH-specific activity of three postgastrula stages before and after a 20-min heat shock at 37°C. In each experiment, the heat-shock sample exhibits a significant increase in GAPDH-specific activity relative to the control sample. Heat-shocked neurulae and later developmental stages show variable specific activity ranging from 80% (a 20% decrease in specific activity) to 210% of control-specific activity.

By immunoblotting techniques, we correlated the increase in specific activity with the accumulation of GAPDH. Rabbit antiserum against yeast GAPDH (Sigma Chemical Co.) was prepared as described in Materials and Methods. The antiserum recognizes a group of four proteins of ~35 kD on a one-dimensional SDS polyacrylamide gel. Fig. 2 *b* shows the 35-kD region of an immunoblot of equal amounts of protein from control and heat-shocked neurulae. The band showing the greatest increase in intensity after heat shock comigrates with purified rabbit muscle GAPDH (Sigma Chemical Co.) and hsp35 (see below). No cross-reactivity of the preimmune serum is detected against either purified rabbit muscle GAPDH or any *Xenopus* protein (Fig. 2 *c*). The apparent increase in GAPDH-specific activity could be due, in part, to generalized protein degradation during heat shock. This is unlikely, however, because we have not detected any decrease in the concentration of protein in heat-shocked embryos or any change in the protein profiles of heat-shocked embryos when analyzed by PAGE and Coomassie Blue staining (data not shown). This observation suggests that GAPDH accumulation and the subsequent increases in specific activity are either due to an increase in GAPDH stability or heat-induced synthesis of the enzyme. Several lines of experimental evidence suggest the latter possibility (see below).

Level of a Specific Isozyme of GAPDH Increases during Heat Shock

When extracts of control and heat-shocked embryos are assayed at ambient temperature (22°C), the heat shock–induced increase in GAPDH-specific activity is negligible (Fig. 3). As the assay temperature is increased, however, the heat-shock sample shows a progressively larger increase in specific activity over the control embryo sample. The increase is greatest at the temperature used to heat shock the embryos (37°C) and begins to decrease sharply at temperatures greater than 42°C. One possible interpretation of these data is that there is an increase in the level of an isozyme of GAPDH in heat-shocked embryos that exhibits a narrow temperature range of activity centered around the heat shock temperature.

To examine the possibility that a specific isozyme of GAPDH increases during heat shock, we analyzed the isozymes of GAPDH in *Xenopus* embryos by separating extracts on nondenaturing 5% polyacrylamide gels. Dramatic evidence of the accumulation of an isozyme of GAPDH is observed in coenocytic embryos. For this experiment, the coenocytic embryos were used when their untreated sibling embryos had reached the dorsal lip gastrula stage. The newly synthesized proteins of the coenocytic embryos were labeled with [^{35}S]methionine during heat shock and separated by nondenaturing gel electrophoresis (Fig. 4). Fig. 4 *a* shows the GAPDH activity-staining pattern of control and heat-shock samples, and Fig. 4 *b* shows the corresponding fluorograph of the same gel. The control sample displays only one isozyme of GAPDH activity, whereas two isozymes are detected in the heat-shock sample. The fluorograph indicates that a doublet of labeled proteins is located at the same position on the gel as the heat shock–specific isozyme (arrows marked by *A*). The upper band of the doublet is an hsp. These results imply that the heat-shocked embryos have enhanced the synthesis of a specific isozyme of GAPDH. However, to compare the relative protein synthesis by control and heat-shocked embryos, equal acid precipitable cpm of ^{35}S were loaded onto each lane of the gel. Heat-shocked embryos occasionally incorporate less [^{35}S]methionine into protein than control embryos. Consequently, a possible explanation for the appearance of heat shock–specific GAPDH staining is that when equal acid precipitable counts were loaded onto each lane of this gel, the heat-shocked lane received proportionately more protein. However, we have never detected the heat shock–specific isozyme of GAPDH in control samples, regardless of the concentration of protein loaded (data not shown). This does not demonstrate that this isozyme is missing in control samples, but it is certainly below the level of detection afforded by enzyme activity staining. In addition, both control and heat-shocked coenocytic embryos have

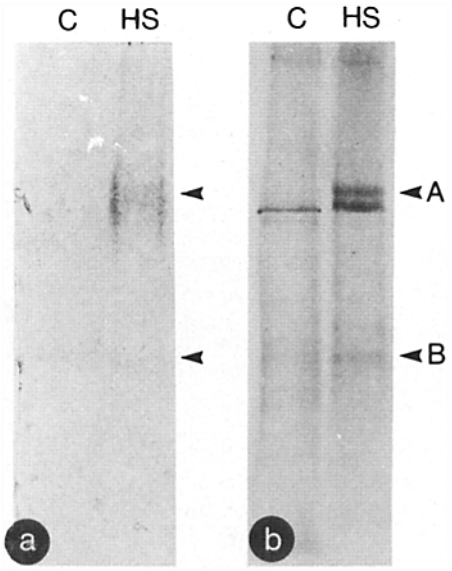

Figure 4. Heat-shocked coenocytic embryos show increases of an isozyme of GAPDH. Comparison of the GAPDH enzyme activity staining pattern (*a*) and fluorograph (*b*) of control (*C*) and heat-shocked (*HS*) coenocytic embryo proteins separated on a nondenaturing polyacrylamide gel. The heat-shocked extracts have a unique band of enzyme activity, which corresponds to the position of a heat-shock protein (*A arrowheads*). Both samples show the enzyme activity staining of second isozyme of GAPDH (*B arrowheads*). The coenocytic embryos were heat shocked when sibling normal embryos had reached the dorsal lip stage of development. Equal acid precipitable cpm were loaded for each lane.

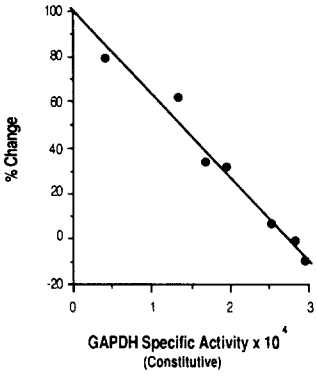

Figure 5. Increases in GAPDH-specific activity are inversely proportional to the level of constitutive GAPDH-specific activity. A graph showing the plot of seven experiments comparing the constitutive level of GAPDH-specific activity and the percent increase in activity of the corresponding heat-shocked sample. Included with the plotted points is the calculated best fit line. There is significant correlation between constitutive GAPDH-specific activity and the subsequent change induced by heat shock (Spearman test, $p < 0.001$). These data are from experiments using neurulae and early larval stages.

comparable staining for the anodal isozyme (Fig. 4, arrows marked by *B*), indicating that this form of GAPDH is present in comparable amounts in both control and heat-shock samples. This condition is representative of the results we obtain when equal amounts of protein are loaded onto nondenaturing gels (data not shown). Our results with coenocytic embryos are similar to results obtained from heat-shocked neurulae, indicating that this phenomenon is not unique to coenocytic embryos. Coenocytic embryos were used in this experiment, rather than neurulae, since hsp35 synthesis (which has many of the properties of GAPDH) is more consistent and more intense than in neurulae. Otherwise, the pattern of heat-shock protein synthesis in coenocytic embryos is comparable to that of neurulae (data not shown).

Increase in GAPDH-specific Activity Is Inversely Correlated with the Constitutive Levels of GAPDH-specific Activity

As mentioned above, the heat shock-induced increase in GAPDH-specific activity is variable. We have observed a trend in the stimulation of GAPDH activity, however. Fig. 5 shows a plot of constitutive GAPDH-specific activity versus the percentage change in activity measured after a 20-min heat shock at 37°C in seven experiments using *Xenopus* neurulae or larvae. It is apparent from this figure that embryos with low levels of constitutive GAPDH-specific activity have large percentage increases in specific activity after heat shock. In contrast, embryos with high levels of constitutive GAPDH activity show little or no increase (and occasionally a decrease) in enzyme activity after heat shock. Embryos may, therefore, have a threshold of GAPDH activity below which they begin to accumulate more of the enzyme during heat shock. This may reflect a dependence on an absolute level of enzyme activity during heat shock.

GAPDH Is Related to the Xenopus 35-kD Heat-Shock Protein

The evidence presented above shows that heat-shocked embryos accumulate GAPDH during heat shock. Accumulation may be due to an increase in the half-life of the GAPDH enzyme relative to other proteins, or it may be due, at least in part, to de novo synthesis of the enzyme. The comigration of an hsp with the heat shock–specific isozyme of GAPDH suggests that this isozyme is being synthesized during heat shock. This possibility is particularly enticing because hsp35 comigrates with GAPDH in one-dimensional polyacrylamide gels. Preliminary data (not shown) also indicates that hsp35 comigrates with rabbit muscle GAPDH in two-dimensional polyacrylamide gels. We have investigated the possibility that hsp35 is a subunit of GAPDH. In our first set of experiments, we correlated the increase of GAPDH-specific activity with the synthesis of hsp35. Both the synthesis of hsp35 and the increase in GAPDH-specific activity are variable, and often hsp35 is not detected in heat-shocked embryos. We compared the appearance of hsp35 with the increase in GAPDH-specific activity in embryos from different female frogs. Eggs from eight females were fertilized with the sperm of one male, and the embryos were grown to the neurula stage. Embryos were heat shocked and prepared for enzyme assays or were labeled with [^{35}S]methionine during heat shock. Fig. 6 shows the results from two spawnings of eggs. Hsp35 synthesis is only detected in the embryos with the relatively large increase in GAPDH-specific activity. The results from the other spawnings are consistent with this observation.

We have also observed a similar correlation between the synthesis of hsp35 and increases in GAPDH-specific activity in the cells of the animal and vegetal hemispheres of heat-shocked early gastrulae. As mentioned in the introduction, hsp35 synthesis appears to be restricted to the cells of the vegetal hemisphere, which coincides with the acquisition of

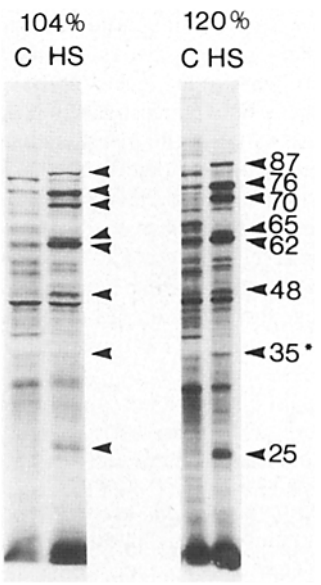

Figure 6. Hsp35 synthesis correlates with increases in GAPDH-specific activity. Fluorograph of [^{35}S]methionine-labeled proteins of control (*C*) and heat-shocked (*HS*) neurulae from two separate spawnings of eggs separated on a SDS 10% polyacrylamide gel. Samples of the same neurulae were also tested for heat shock–induced changes in GAPDH-specific activity. The change in specific activity is shown at the top of each set of samples as a percent of control GAPDH activity (i.e., control activity = 100%). The molecular masses of the hsps are shown in kilodaltons. Hsp35 (*asterisk*) is detected in the sample with the relatively high increase in GAPDH-specific activity. Equal acid precipitable cpm were loaded for each lane.

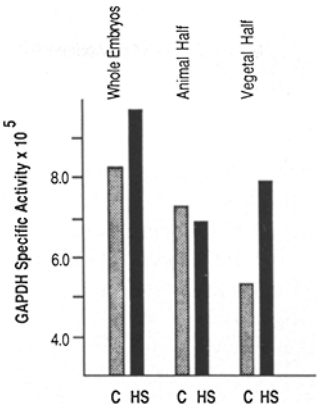

Figure 7. Histograms of GAPDH-specific activity in control (*C*) and heat-shocked (*HS*) whole embryos and in animal and vegetal halves. The embryos were heat-shocked at 35°C for 60 min. The whole embryos exhibit a significant increase in GAPDH-specific activity after heat shock (Mann Whitney U-test, $p = 0.05$). The heat-shocked animal halves show no increase in specific activity relative to control ($p > 0.10$), but the heat-shocked vegetal halves show a significant increase in activity ($p = 0.05$). The restriction of heat-induced increases in GAPDH-specific activity to the vegetal halves may be due to significantly lower levels of constitutive GAPDH-specific activity in these cells over those of the animal halves ($p = 0.05$).

thermotolerance by these cells (39). Similarly, heat-induced increases in GAPDH-specific activity appear to be restricted to these cells. Fig. 7 shows the results of an experiment in which we cut early gastrulae into animal and vegetal halves with a pair of microscissors, heat shocked the two halves for 60 min at 35°C, and then processed them for GAPDH enzyme assays. These heat-shock conditions are similar to those used in the labeling experiments reported earlier (39). Animal halves exhibit significantly more constitutive GAPDH-specific activity than the vegetal halves. This difference in constitutive specific activity reflects the predicted changes in specific activity after heat shock: animal halves do not show a significant change in specific activity, whereas vegetal halves show an increase. In the experiment shown here, the total specific activity of the dissected embryos was determined to compare it to the specific activity measured for the whole embryos. The total constitutive specific activity from the dissected embryos is not significantly different from that of the whole embryos (Mann Whitney U-test, $p > 0.20$), indicating that cutting the embryos does not alter the apparent levels of enzyme activity of the whole embryo. However, the total heat-induced change in GAPDH-specific activity of the dissected embryos was slightly, but significantly, lower than the heat-induced change in specific activity of the intact embryos ($p < 0.05$), indicating that cutting the embryos may reduce their ability to muster a maximal heat shock response. It is also important to note that we have never detected heat-induced increases in GAPDH-specific activity in early or mid-cell blastulae (data not shown). Hsp35 synthesis has also never been detected in embryos at these stages of development (20, 39).

To analyze further whether hsp35 and GAPDH are the same protein, we also digested hsp35 with N′-chlorosuccinimide, which cleaves proteins at tryptophanyl peptide bonds. As described in Materials and Methods, the peptide map of hsp35 was analyzed by fluorography, and the molecular masses of the resulting fragments were determined based on their migration relative to molecular mass standards. These molecular masses are shown in Table I and are compared to the relative molecular masses of fragments generated from the N′-chlorosuccinimide digestion of rabbit muscle GAPDH and the calculated molecular masses of fragments predicted by the digestion of chicken muscle GAPDH based on its complete amino acid sequence as reported by

Table I. Comparison of the Molecular Masses of the N′-Chlorosuccinimide Digestion Products of Hsp35 and GAPDH

Relative molecular mass		Predicted molecular mass	
Hsp 35	GAPDH* (rabbit)	GAPDH (chicken)	Amino acid length
kD		*kD*	
32	33	35	333
31	29	32.3	308
27	27	25.7	245
24	23	23.7	226
20	17	20.4	194
15	14	14.6	139
–‡	12§	12.3‡	117
13		11.5§	109
		8.9§	85
		2.3§	22

* The fragment sizes of rabbit muscle GAPDH were determined from a 12% polyacrylamide gel, rather than a 15% gel used for hsp35.

‡ No methionine residues are present in this fragment of chicken muscle GAPDH.

§ The N′-chlorosuccinimide digestion of rabbit muscle GAPDH and hsp35 yields 7 fragments, whereas the predicted number of fragments for GAPDH is 10. It is unlikely that the gel system used for these experiments resolves the 2.3-kD fragment. It is also possible that the 3 fragments of 12.3, 11.5, and 8.9 kD migrate as one band. The only discrepancy between the fragment sizes of GAPDH and hsp35 is in the first fragment. This fragment is predicted to be undigested GAPDH monomer and it comigrates with undigested GAPDH for both rabbit muscle GAPDH and hsp35. Presumably the discrepancy in the determination of the first fragment of GAPDH is due to error produced by extrapolating this molecular mass from standards. The molecular masses of the predicted fragments of chicken muscle GAPDH were estimated by multiplying the length of each fragment (in amino acids) by a constant of 0.105 kD per amino acid.

Panabières et al. (41). It is apparent that the N'-chlorosuccinimide digestion of GAPDH and hsp35 produces fragments of similar sizes, suggesting that they are related proteins.

Discussion

Our evidence shows that *Xenopus* neurulae can respond to heat shock by accumulating an isozyme of GAPDH, which occurs concomitantly with heat shock–induced increases in GAPDH-specific activity. This isozyme appears to exhibit a narrow temperature range of optimum activity, which is centered around the temperature used to heat shock the embryos. In one-dimensional SDS polyacrylamide gels, this enzyme comigrates with the previously described hsp35, suggesting that GAPDH is de novo synthesized during heat shock. The correlation between GAPDH and hsp35 is further reinforced by the similarity between the peptide maps of rabbit muscle and chicken muscle GAPDH and hsp35. Both the heat shock–induced synthesis of hsp35 and the increases in GAPDH-specific activity are variable. There is a trend in the increases in GAPDH-specific activity, however, in that the increase in specific activity is inversely correlated with the level of constitutive GAPDH-specific activity. Accordingly, hsp35 synthesis is only detected in samples that exhibit relatively large heat-induced increases in GAPDH activity. These data suggest that the constitutive level of GAPDH tightly controls the heat shock-stimulated accumulation of GAPDH. Currently, we are examining at what level of control the expression of GAPDH is regulated (i.e., transcriptionally or translationally). Further study is required to determine how the constitutive levels of GAPDH influence or determine this regulation.

Roles of GAPDH and Glycolysis during Heat Shock

As mentioned in the introduction, cells appear to consume ATP during heat shock and may be forced to rely on glycolysis to supply more of it. We report here that heat-shocked *Xenopus* embryos exhibit changes in energy metabolism, including higher rates of ATP hydrolysis relative to control embryos and the accumulation of lactate, which indicates that they may rely on anaerobic glycolysis to synthesize more ATP during heat shock. We suggest that the embryos meet this demand for anaerobic glycolysis by increasing the levels of some glycolytic enzymes.

A role for glycolysis in heat-shocked *Xenopus* embryos would be further strengthened by evidence showing that heat-shocked embryos accumulate and/or synthesize other glycolytic enzymes as well. Our laboratory has evidence that a second glycolytic enzyme, pyruvate kinase, is hsp62 (Nickells, R. W., and L. W. Browder, manuscript in preparation). Although we have not yet examined for increases in any other glycolytic enzymes, proteins similar to the *Xenopus* hsps are synthesized by maize seedlings that are placed under anaerobic conditions (46). These "anaerobic proteins" have molecular masses of 87, 77, 65, 64, 55, 45, 40, and 35.5–31.5 kD, which are very similar to the molecular masses of the *Xenopus* hsps (see Fig. 6). The role of anaerobic proteins appears to be in stimulating an increase in the rate of anaerobic glycolysis to enhance the survival of anaerobically stressed seedlings. This function is confirmed by the identification of some of the anaerobic proteins as the enzymes alcohol dehydrogenase (14, 45, 53), fructose-1,6-diphosphate aldolase (24), pyruvate decarboxylase (53), and glucose phosphate isomerase (25), which are involved in carbohydrate metabolism.

An important consideration when assigning a thermoprotective role to glycolysis is that only yeast cells and now *Xenopus* embryos are known to increase the levels of glycolytic enzymes during heat shock. Other investigators have not observed increases in the activities of the glycolytic enzymes in some heat-shocked cells, even though heat shock is known to stimulate the accumulation of lactate in these cells (26). A possible explanation for this may be that in these cell types, the preexisting levels of glycolytic enzymes are able to cope with the ATP demand during heat shock. This would be analogous to heat-shocked *Xenopus* embryos that do not accumulate GAPDH because of high constitutive levels of GAPDH activity.

A similar argument may explain the restricted synthesis of hsp35 to the vegetal hemisphere cells of the early gastrula observed previously (39). Animal hemisphere cells exhibit greater constitutive GAPDH-specific activity than the vegetal hemisphere cells. Presumably, these cells already have sufficient levels of the enzyme and therefore do not require an increase during heat shock. The vegetal hemisphere cells, however, with their significantly lower constitutive level of GAPDH activity, do exhibit increases during thermal stress. This increase in activity correlates with the region-specific synthesis of hsp35 in these cells. The existence of regional differences in GAPDH-specific activity is not surprising. Amphibian embryos have animal > vegetal gradients of glycogen granules, mitochondria, and O_2 consumption, suggesting an animal > vegetal gradient in carbohydrate metabolism (4, 5). This metabolic activity is especially pronounced at the onset of gastrulation when O_2 consumption dramatically increases (48) and glycogen stores dramatically decrease (18).

We have presented evidence that *Xenopus* embryos accumulate an isozyme of GAPDH during heat shock and that this accumulation may be partly due to its de novo synthesis. The accumulation of this enzyme and the documented observations of heat-induced alterations in energy metabolism in other cell types raise the possibility that glycolysis is a major component of short-term thermotolerance in many cell types. Thermotolerance, therefore, may have two separate components: an energy requirement and structural repair. This latter component is the presumed thermoprotective role of hsps; i.e., minimizing or reversing heat damage. For example, hsp70 has been postulated to refold proteins denatured by heat shock (43). The thermoprotective action of glycolytic enzymes may not be to protect the cell from heat damage, but rather to provide needed energy to the cell during heat shock. In the case of *Xenopus* embryos, the accumulation of glycolytic enzymes is ultimately controlled by the metabolic state of the cell and may only be stimulated when a certain threshold of some substance (perhaps a glycolytic intermediate) has been reached.

The authors would like to thank Dr. M. Bentley, Dr. J. Dulson, Dr. M. Pollock, Dr. J. Heikkila, and Dr. L. Gedamu for helpful discussions of experiments and suggestions regarding the manuscript.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the University of Calgary to L. W. Browder and an Alberta Heritage Foundation for Medical Research

research allowance to R. W. Nickells. R. W. Nickells is also the recipient of a Natural Sciences and Engineering Research Council of Canada postgraduate scholarship.

Received for publication 22 February 1988, and in revised form 29 July 1988.

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